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Jou Journal of Lab Animal Research. 2023; 2(4): 16-22.

DOI: 10.58803/jlar.v2i4.20

http://jlar.rovedar.com/

Original Article

Ameliorative Effects of Vitamin C and Methanolic Extract of Broccoli on Cyclophosphamide-induced Poisoning in Ovary of Rats

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ARTICLE INFO

Article History:
Received: 21/06/2023
Accepted: 23/07/2023

Keywords:
Antioxidant
Broccoli extract
Cancer
Cyclophosphamide
Ovary
Vitamin C

ABSTRACT

Introduction: Considering the importance of using herbal compounds to reduce the side effects of cyclophosphamide (CPH), the current study aimed to evaluate the effects of broccoli extract and Vitamin C on ovarian poisoning with CPH.
Materials and methods: Four equal groups of 48 adult female Wistar rats were formed. The first group (control) received physiological saline orally. A 200 mg/kg dose of CPH was administered intraperitoneally to the second group. For the third group, CPH was supplemented with 300 mg/kg of Vitamin C, and methanol extract of broccoli 300 mg/kg was used in the fourth group. The serum total antioxidant capacity (TAC), interleukin-1 and tumor necrosis factor alpha (TNFα) and ovarian tissue glutathione peroxidase (GPx), superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT), were measured. At the end of the study, the ovarian tissue was cut and stained for histopathological investigations.
Results: Ovarian tissue GPx, CAT, and SOD values indicated a significant decrease in the CPH group compared to other groups. In the CPH plus broccoli group, there was a significant decrease in MDA ovarian tissue and IL-1 and TNF-α in serum, compared to the CPH group. There were significant negative changes in ovarian cells of the CPH group, compared to the control and other treatment groups.
Conclusion: The current study suggested that administrating broccoli extract plus CPH could increase the superior antioxidant potential, compared to Vitamin C. This can potentially decrease CPH-induced damage to the ovary of rats, thereby improving their fertility status.

1. Introduction

Cancer is one of the deadliest diseases in all around the world, and lots of treatments have been suggested to combat it. The number of women diagnosed with cancer is estimated at 6.6 million annually. This represents 10% of all women under 40 years diagnosed with cancer[1]. Patient survival rates for cancer have significantly increased due to advancements in chemotherapy and other treatments. However, a lot of women have experienced decreased ovarian reserve as a result of chemotherapy[2]. Cyclophosphamide (CPH), an alkylating agent frequently used to treat gynecological cancer, is among the most gonadal toxic medications and can cause early ovarian failure and infertility[3]. As a broad-spectrum antineoplastic medication, the regularly used chemotherapeutic CPH has a variety of effects[4,5]. Apoptosis is caused by CPH through activating both Bax and mitochondrial pathways in developing follicles[6]. Granulosa cells in developing follicles multiply quickly and are extremely sensitive to chemotherapy[7]. Cyclophosphamide destroys the preliminary follicles in the ovary and reduces the follicular reserve[8]. Increased production of oxygen free radicals, particularly reactive oxygen species (ROS), which can have various harmful effects, is one of the effects of CPH[9]. This excessive free radical generation surpasses the capacity of antioxidant mechanisms to counteract them, leading to oxidative stress[10]. Following the initiation of apoptosis in the ovary, key processes include the oxidative stress reactions in the tissue and the decline in antioxidant properties, such as glutathione peroxidase (GPx),

► *Cite this paper as*: Ahmadi Simab P, Raeeszadeh M, Karami N. Ameliorative Effects of Vitamin C and Methanolic Extract of Broccoli on Cyclophosphamide-induced Poisoning in Ovary of Rat. Journal of Lab Animal Research. 2023; 2(4): 16-22. DOI: 10.58803/jlar.v2i4.20

catalase (CAT), and superoxide dismutase (SOD). Malondialdehyde (MDA) can be used to evaluate the peroxidation of lipids[11–13]. The body employs a range of indicators for measuring oxidative stress. The ovary uses indicators, such as glutaredoxin, GPx, nitric oxide, CAT, SOD, lipid peroxides like MDA, reactive molecules to thiobarbituric acid, and total antioxidant capacity (TAC)[14]. Fertility protection is receiving more attention in light of this predicament. One critical step in the subsequent clinical development is identifying a potential protective medication for ovarian function.

The use of chemical and herbal compounds to reduce side effects of infertility and improve reproductive status has been considered when prescribing anticancer drugs[15]. Consuming foods high in antioxidants can reduce the negative effects of ROS[16]. Vitamins, polyphenols, and carotenoids are among the antioxidants included in the therapeutic herb extracts[17]. Removing free radicals is made possible by vitamins, which are crucial antioxidants[18]. Among these, Vitamin C, also known as ascorbic acid, is present in most biological systems and has the highest plasma antioxidant properties in the ovary and a strong ROS scavenger effectiveness[19]. Therefore, Vitamin C consumption can reduce oxidative damage[20].

Broccoli (*Brassica oleracea* var. Italica) belongs to the Cruciferous family. It is a significant source of minerals, ascorbic acid, carotenoids, flavonoids, cinnamic acid derivatives, glucosinolates, and flavonoids[21]. The primary components of the antioxidant potential of cruciferous vegetables are tocopherols and carotenoids[22]. Endogenous antioxidants, such as tocopherol, ascorbic acid, carotenoids, and phenolics, are also abundant in broccoli[23]. In each stage of plant development, there are distinct combinations of antioxidants. Phenolic molecules are one of the primary factors in these veggies' antioxidant properties[24,25].

Due to the unique characteristics of broccoli extract and Vitamin C in terms of antioxidant levels, they can be used effective composition in oxidative damage[26]. Given the significance of CPH ovarian side effects, the current study aimed to compare the effects of Vitamin C and broccoli extract on ovarian poisoning with CPH.

2. Materials and Methods

2.1. Ethical approval

The Animal Care Committee at the Islamic Azad University of Sananda, Sanandaj, Iran, approved all procedures. Both specific international law and the standards of laboratory animal care were upheld.

2.2. Animals

A total of 48 female Wistar rats from the Pasteur Institute of Iran were used in the current study. The rats were within the weight range of 220-250 grams, and age range of 6-8 weeks. Throughout the study, all animals were kept in regulated environmental settings (temperature of 20-21°C, long light/dark periods with a 12-hour cycle of light and darkness), with free access to clean water and food (Behparvar Company, Iran). The study was performed after 7 days of rats' environmental adaptation.

2.3. Study design

Four equal groups of rats were randomly assigned. The first group was control (C) and received physiological saline orally. Cyclophosphamide was administered intraperitoneally to the second group once a week for 3 consecutive days at 200 mg/kg. The third group received CPH plus Vitamin C at 300 mg/kg/day. Finally, CPH plus methanol extract of broccoli was administered to the fourth group. The study lasted 21 days. Intraperitoneal injections of ketamine (100 mg/kg) and xylazine (10 mg/kg) were used to anesthetize the rats. The current study measured TAC with fluorescence recovery after the photobleaching method under general anesthesia on serum samples taken from animal hearts. Further experiments were performed using ELIZA technique to measure interleukin 1 and tumor necrosis factor-alpha (two proinflammatory cytokines). Thermometric analysis was carried out on the right ovarian tissue with the use of a ZELLBIO calorimetric kit in order to determine the levels of GPx, CAT, SOD, and MDA[27]. After separating the left ovary, it was fixed in 10% formalin for 24 hours.

2.4. Chemical compound preparation

The current investigation used CPH (CAS number: 605-19-2) and pure Vitamin C powder (CAS number: 50-81-7) with molecular weights of 279.10 g/mol and more than 99.5% purity (Sigma-Aldrich, USA).

2.5. Preparation of methanolic extract from broccoli

Broccoli (*Brassica olaracea*) was collected in April 2021 from a farm in Hamadan, Iran, and authenticated by the Kurdistan University Herbarium Center, Iran (No: 30073). Broccoli heads were cut into tiny florets, and tough stalks were removed. During overnight storage at 25°C, 200 mL of 80% methanol was mixed with 2 grams of dried florets. The methanol was then extracted from the solution using an IKA-RV 8 vacuum rotary machine after the solution was using German Whatman paper. The resultant material was baked dry and lyophilized to produce a powder. After being dissolved in sterile distilled water at a concentration of 0.1 g/mL, this powder was gavage-injected into rats daily at a dose of 300 mg/kg.

2.6. Total antioxidant capacity level measurement

In order to measure the TAC, the FRAP method was used. This method quantifies the ability of ferric ions to be

reduced by plasma. A blue color was produced at 593 nm when the Fe III-TPTZ complex was reduced to Fe II at an acidic pH. Total antioxidant capacity values were calculated using a concentration range of 100-1000 mol/L and a standard diagram[28].

2.7. Investigation of serum pro-inflammatory cytokines

An enzyme-linked immunosorbent assay kit (ELISA) from the USCN (Chinese) and constructor methods were used to evaluate serum levels of IL-1 and TNFα. The results were subsequently expressed in a reading of pg/mL[29].

2.8. Catalase and superoxide dismutase measurements

Measurement of catalase activity was carried out using the Claiborne method[30]. This was achieved by mixing the sample up to a volume of 3 ml with 0.09 M H2O2, 0.1 M phosphate buffer, and PMS (10%), with the components proportioned correctly. Subsequently, an absorption spectrophotometer was utilized to measure the absorbance at 240 nm after allowing the mixture to stand for 30 seconds.

2.9. Malondialdehyde and glutathione peroxidase measurements

To quantify the malondialdehyde level, a 10% TAC solution was added in a volume twice that of the homogenized ovarian tissue, followed by intense vortexing and centrifugation at 2500 rpm for 10 minutes. Then, in the same volume as the supernatant solution, TBA 0.67% was added. The mixture was then immersed in a water bath at 100 degrees for an hour to determine the amount of malondialdehyde present. This study used Paglia and Valentine's method for measuring glutathione peroxidase. Using 340 nm light absorption, glutathione (GSH) is measured by its oxidation reaction via cumene hydroperoxide[31].

2.10. Investigation of Histological ovary

At least five-thick microtome sections of ovarian tissue were examined for histological examination of primary, secondary, graph, corpus luteum, and atretic follicles. Using a light microscope (Nikon 3200, Japan), these sections were stained with hematoxylin and eosin (H & E).

2.11. Data analysis

To present the study's findings, mean standard errors and standard deviations were used. In order to compare mean values between groups, a one-way ANOVA and a Tukey post-hoc test were performed. Statistical analyses were conducted using SPSS 23 software with a significance level of $p < 0.05$.

3. Results

3.1. Glutathione peroxidase, superoxide dismutase, malondialdehyde, and catalase concentration in the studied groups

According to Table 1, the highest concentration of antioxidant enzymes GPx, SOD, and CAT were seen in the control group and the significantly lowest in the CHP group ($p < 0.05$). In the groups that received broccoli extract, there was an increase in enzyme concentrations, with GPx showing a non-significant difference, compared to the control group ($p > 0.05$). Malondialdehyde values were significantly lower in the control group ($p < 0.05$) and significantly higher in the CHP group ($p < 0.05$). The malondialdehyde levels in the broccoli and Vitamin C groups were significant, compared to the control group ($p < 0.05$).

3.2. Total antioxidant capacity, interleukin-1, and tumor necrosis factor alpha concentration in the serum of the studied groups

As shown in Figure 1, the control group had significantly higher antioxidant capacity levels than the CHP group ($p < 0.05$). There was a significant difference between the CHP and control groups regarding the levels of IL-1 and TNF ($p < 0.05$). Vitamin C therapy and broccoli therapy did not significantly influence the levels of TCA, IL-1, and TNF ($p > 0.05$).

3.3. Histology of ovarian tissue

According to the histological findings, secondary follicles and primary follicles were significantly smaller

Table 1. Ovarian tissue oxidative stress parameters of experimental groups

Parameters	Groups			
	C	CHP	CHP+BC	CHP+VC
GPx U/g tissue	180.47 ± 12.38^{c}	57.38 ± 10.25^{a}	120.28 ± 23.46^{b}	93.27 ± 9.28^{b}
SOD U/g tissue	31.28 ± 8.56^{c}	12.28 ± 4.25^{a}	26.37 ± 10.11^{b}	22.15 ± 10^{b}
CAT U/g tissue	27.39 ± 3.48^{c}	6.79 ± 1.25^{a}	18.23 ± 9.90^{b}	20.26 ± 7.38^{b}
MDA nmol/g tissue	1.65 ± 0.58^{a}	15.34 ± 2.38^{c}	3.49 ± 0.27^{b}	7.47 ± 3.46^{b}

C: Control, CPH: Cyclophosphamide, CPH+BC: Cyclophosphamide plus methanol extract of Broccoli at a dose of 300 mg/kg, CHP+VC: Cyclophosphamide plus Vitamin C at a dose of 300 mg/kg, GPx: Glutathione peroxidase, CAT: Catalase, SOD: Superoxide dismutase, MDA: Malondialdehyde. ^{a,b,c,} Different superscript letters in the same rows mean significant differences ($p < 0.05$).

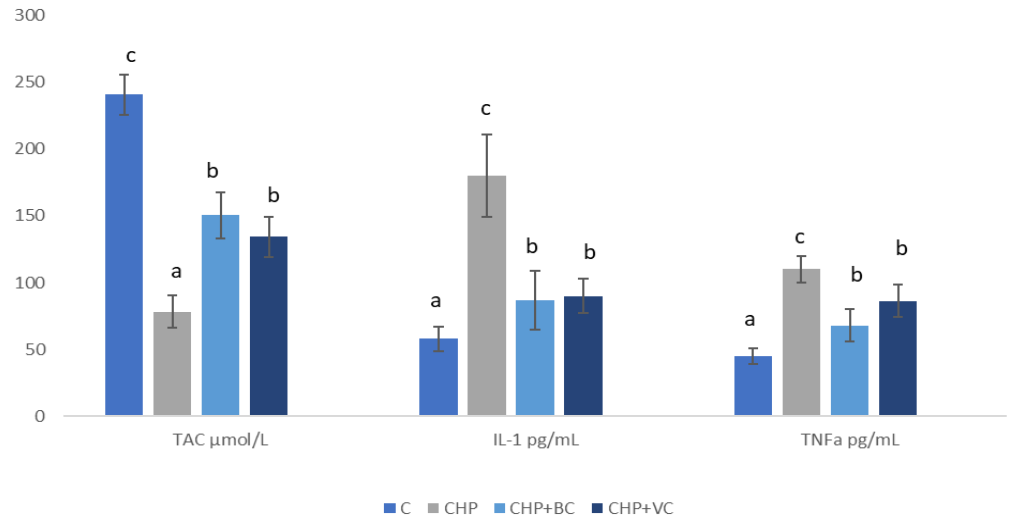

Figure 1. Serum TAC, IL-1, and TNFα levels of experimental groups. C: Control, CPH: Cyclophosphamide, CPH+BC: Cyclophosphamide plus methanol extract of broccoli at a dose of 300 mg/kg, CHP+VC: Cyclophosphamide plus Vitamin C at a dose of 300 mg/kg. TCA: Total antioxidant capacity, IL-1: Interleukin -1, TNFα: Tumor necrosis factor-alpha, [a,b,c,] Different superscript letters in the same group mean significant differences ($p < 0.05$).

in the CHP group than in the control group. There was a significant increase in the number of graft follicles in the CHP plus broccoli group, compared to the CHP group. The number of follicles that were found to be atretic increased in the CHP group in comparison with other groups (Figure 2).

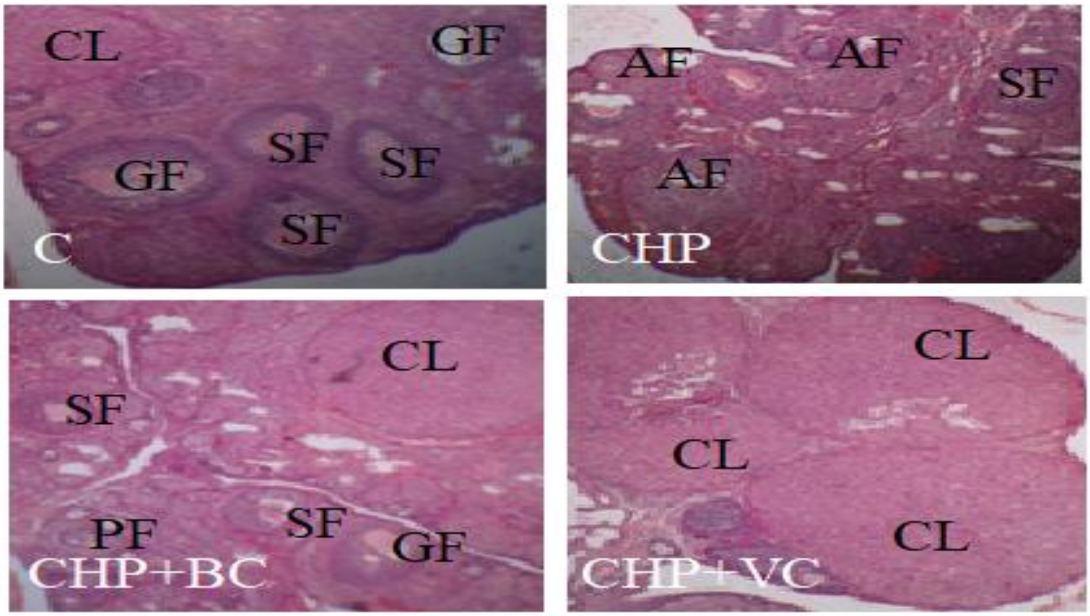

Figure 2. Ovarian microscopic sections in the studied groups. PF: Primary follicle; SF: Secondary follicle; GF: graph follicle; Cl: corpus luteum; AF: Atretic follicle (Hematoxylin-eosin staining, ×400).

4. Discussion

This interventional study examined the effects of Vitamin C and a broccoli herbal extract in minimizing the negative effects of CPH, which has been associated with side effects, particularly in ovarian tissue.

The rate of lipid peroxidation is accelerated by the fact that lipids, particularly unsaturated fatty acids in the cell membrane, are among the biological components most vulnerable to ROS attack[32]. The major marker of unsaturated fatty acid oxidation and an indication of oxidative stress in the etiology of many diseases is malondialdehyde[33]. The findings of the current study indicated that the MDA level increased significantly due to the administration of CPH at a dose of 200 mg/kg. The lowest level of MDA was observed in the broccoli group at a dose of 300 mg/kg, indicating its antioxidant properties of this plant. The concentrations of the antioxidant enzymes GPx and TAC increased under the treatment of Vitamin C and broccoli herbal extract, demonstrating he beneficial antioxidant effects of these compounds. Furthermore, the reduction in IL-1 and TNF

proinflammatory biomarkers in the groups treated with Vitamin C and broccoli herbal extract, compared to the CPH group, indicated the interaction between these two antioxidants and anti-inflammatory capabilities in experimental CPH-induced ovarian damage. The growth of primary and secondary follicles and the decrease of atretic follicles in the Vitamin C and broccoli herbal extract groups were also confirmed by a microscopic examination of the ovarian follicles. Ovulation involves a physiological mechanism called inflammation. Nevertheless, unchecked inflammation negatively impacts ovulation and hormone production[34,35]. Reactive oxygen species also rise during steroidogenesis and ovulation in the ovary. Detoxification of ROS is, therefore, necessary for oocyte maturation and fetal development. Reactive oxygen species harm ovarian function through lipid peroxidation and MDA formation, [36].

Lipid peroxidation in the plasma membrane of luteal cells damages gonadotropin receptors and inhibits steroidogenesis in the corpus luteum[37]. In line with other studies, the findings revealed that the CHP plus broccoli group had considerably more transplant follicles than the CHP group in terms of histological status[38]. By reducing the oxidative damage and inflammation brought on by CPH, antioxidants significantly impact follicle formation and healthy ovarian function.

Various studies have shown that mice fed spirulina are more resistant to ovarian-damaging effects of CPH[39]. It has been shown that spirulina protects rats against the damaging effects of CPH, based on early investigations[40]. Based on the findings of a previous study conducted on women receiving CPH therapy, where their diet was supplemented with alpha-LA and omega-3 fatty acids, it has been suggested that there may be a potential benefit in preventing CPH-induced infertility in women of childbearing age[41]. There is also evidence that quercetin and rosuvastatin have a protective effect against ovarian toxicity and POF caused by CPH, without compromising their anti-tumor efficacy, as demonstrated in a previous study[42]. In addition to their influence on follicular development and angiogenesis, cytokines could facilitate indirect nutrient delivery[43,44]. This intervention also reduced the concentrations of proinflammatory markers (IL-1 and TNFα) in the groups of herbal extract of broccoli and Vitamin C, compared to CPH.

According to histological findings, CPH increased atretic follicles while decreasing ovarian follicles. The immune system was modulated, the oxidative balance was maintained, and damaged follicles were supported in growing with the help of Vitamin C and broccoli herbal extract. Thus, the results of the current investigation were supported[45]. In this regard, broccoli with effective vitamins C and E, sulforaphane, and phytoestrogen compounds control these complications and improve ovarian function. At the same time, Vitamin C as a single antioxidant showed lower comparative effects than broccoli in the treated groups[46].

5. Conclusion

There is strong evidence that certain antioxidants, such as Vitamin C and broccoli herbal extract, significantly suppress the production of MDA while at the same time increasing the production of TAC and GPx in CPH patients. Despite this, serum levels of TNF and IL-1 were still low but reached balance by the end of the study. By increasing the average number of ovarian follicles, as a result of these biochemical processes, the uterus fertility is enhanced. There was no difference in performance between Vitamin C and broccoli herbal extract in terms of reducing the negative effects of CPH on the ovary caused by CPH. The oxidative stress and proinflammatory indicators found in ovarian tissue and their molecular responses may provide new avenues for research to advance our findings.

Declarations

Competing interests

There are no stated conflicts of interest by the authors.

Authors' contributions

Mahdeih Raeeszadeh, Negin Karami, and Pouria Ahmadi Simab designed the study and performed the sampling, statistical analysis, and practical procedures. Pouria Ahmadi Simab wrote the draft of the manuscript and removed language errors. All authors reviewed and approved the final version of the manuscript for publication in the present journal.

Funding

No funding was received for conducting this study.

Ethical considerations

All of the writers have checked for plagiarism, consent to publish, misconduct, data fabrication or falsification, duplication of publication or submission, and redundancy.

Availability of data and materials

The data presented in this study are available on request from the corresponding author.

Acknowledgments

The authors thank the Faculty of Veterinary Medicine, Islamic Azad University of Sanandaj, Iran, for providing the material and technical base/assistance in conducting experimental work.

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